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Research Article

Clustering Analysis and Genome Inference of Pisang Raja Local Cultivars (*Musa* spp.) from Java Island by *Random Amplified Polymorphic DNA* (RAPD) Marker

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ABSTRACT

Pisang Raja is an important local banana cultivar in the economy and cultural life in Indonesia, especially at Java. There are many Pisang Raja cultivars found on Java Island with various local names in each region, resulted in problems on taxonomic identification and grouping. Conventional research for grouping banana cultivars is still using morphological characters but considered inaccurate because of its subjectivity. This study aims to analyze the genetic diversity, grouping, and genome estimation of 13 local cultivars of Pisang Raja based on molecular approach using RAPD markers (OPA primers 1-20). Clustering and Principal Coordinates Analysis were performed to the amplified products using Paleontological Statistics (PAST) application version 3.15. Results showed that there were 12 primers which successfully amplified and produced DNA polymorphic bands in Pisang Raja, specifically OPA 1, OPA 2, OPA 3, OPA 4, OPA 5, OPA 8, OPA 16, OPA 17, OPA 18, OPA 19, and OPA 20. Pisang Raja cultivars considered have high genetic diversity, indicated by high polymorphic bands (95.17%) and low similarity coefficient values (0.2-0.6). Clustering and PCo analysis resulted in 3 clusters following its genomic group consist of AAA, AAB and ABB genomes, with Pisang Raja Bali as an outgroup (ABB). However, the separation of each cluster for genome inference was unclear. Cluster 1 consists of Pisang Raja Madu (AAB) and Raja Sereh (AAB). Cluster 2 consists of AAA and AAB genomes; includes Pisang Raja Jambe (AAA), Raja Kriyak (AAA), Raja Kutuk (AAB), Raja Brentel (AAB), Raja Seribu (AAB), and Raja Lini (AAB). Cluster 3 consists of AAA and AAB genomes, includes Pisang Raja Kisto (AAA), Raja Delima (AAA), Raja Bandung (AAB) and Raja Gareng (AAB). While Pisang Monyet (AAw) and Klutuk Wulung (BBw) as wild relatives were nested in Cluster 2. There were some different results of genome estimation based on RAPD markers compared to morphological characterization, and other molecular techniques. The use of RAPD markers is quite efficient and effective for studying genetic diversity and identifying genomes in bananas.

INTRODUCTION

Bananas (*Musa* spp.) are herbaceous plants in the Musaceae family which widely distributed in tropical and subtropical regions (Simmonds, 1959). Indo-Malesia is the origin center of banana diversity in the world, which then spread to all tropical and subtropical regions in Asia, America, Africa and Australia. (Simmonds and Shepherd, 1955; De

Langhe *et al*., 2009). In Indonesia, the diversity center of bananas is spread across Sulawesi, Sumatra, Madura and including Java (Ochse, 1931). Nowadays, bananas are fruit plant with high value as a horticultural commodity in the world (Ministry of Agriculture, 2016). Indonesia has contributed 5.67% and occupies the sixth position as the world's banana production center. Some regions which contributed to banana production in Indonesia, include East Java (21.87%) West Java (19.22%), Lampung (18.20%), and Central Java (7.68%) (Ministry of Agriculture, 2016). The number of local bananas available in Indonesia is more than 200 cultivars. Some of the popular banana cultivars in Indonesia, viz. Pisang Susu, Kepok, Tanduk, Nangka, Ambon, Raja, etc. (Nasution and Yamada, 2001).

Pisang Raja is one of the popular banana which has an important role in the economy and cultural life in Indonesia, especially at Java Island (Hapsari *et al.,* 2017). In Indonesia, Pisang Raja is widely produced in Java Island, such as in West Java (Sukabumi and Cianjur), Central Java (Demak) and East Java (Lumajang) (Ministry of Agriculture, 2016). There were not less than 40 Pisang Raja local cultivars recorded from several studies of inventory and diversity of bananas in Java Island (Jumari and Pudjoarinto, 2000; Indraswari, 2014; Firdausi *et al.,* 2015; Hapsari *et al.,* 2017; Hapsari *et al.,* 2018). The use of different local names in each region is a problem in the taxonomic nomenclature of Pisang Raja. For example, Pisang Raja Bulu was also known as Raja Madu in Jember, Raja Kul was also known as Raja Talun in Pasuruan and Probolinggo, Raja Pakak was also known as Raja Sepet in Pasuruan, and Raja Sajen was also known as Raja Talun in Malang (Hapsari *et al.,* 2017).

Pisang Raja is a species of hybridized plant which generally has triploid genome characters of AAB, AAA and ABB (Espino *et al*., 1992; Jumari and Pudjoarinto, 2000; Ekasari *et al*., 2012; Hapsari *et al*., 2015a). Those genome characters owned by Pisang Raja was originated from ancestors *Musa acuminata* Colla (A genome contributor) and *Musa balbisiana* Colla (B genome contributor). Further, to overcome the problem of many local names in banana cultivars, Simmonds and Shepherd (1955) proposed the use of genome nomenclature was then approved by a consensus in 1999, which consist of generic name, followed by letter combinations indicating the ploidy and genome sets contributed by their ancestral, followed by the name of cultivars group or the cultivars (Simmonds, 1959; Valmayor *et al*., 2000; INIBAP, 2006).

Genomic composition and various ploidy levels of banana cultivars were first derived from the development of parthenocarpy and sterility. Those events were followed by chromosome restitution and out-crossing of the ancestral both intra and inter -species, then high bananas diversity emerged with various ploidy levels and genomic combination such as AA, AAA, AB, AAB, BB, and ABBB (Simmonds, 1959; Espino *et al*., 1992; Singh *et al*., 2001; Valmayor *et al*., 2000). In determining the genome of banana cultivars can be done by scoring assessment based on morphological characters (Simmonds and Shepherd, 1955; Simmonds, 1959). However, morphological approaches are often inaccurate because it was subjective and can be influenced by environmental factors (Jumari & Pudjoarinto, 2000; Guzow-Krzeminsk *et al*., 2001). Thus, it is necessary to conduct grouping and taxonomy nomenclature analysis based on genotypes with molecular markers.

The approaches through molecular markers were known to have a higher level of accuracy compared to the morphological approach in the identification and grouping of banana cultivars (Williams *et al.,* 1990; Maftuchah, 2001; Rao and Hodgkin, 2002; de Jesus *et al.,* 2013; Hapsari *et al.,* 2015a). One of the molecular markers which often used in research on genetic diversity in plants was *Random Amplified Polymorphic* DNA (RAPD). It is a DNA amplification technique based on *Polymerase Chain Reaction* (PCR) using random single oligonucleotides (primers) to form DNA fragments (Bustaman and Moeljopawiro, 1998; Dayarani and Dhanarajan, 2014). Further, RAPD markers are often used to determine the taxonomy and grouping on banana cultivars in many countries (Uma *et al.,* 2006; Sukartini, 2008; Brown *et al.,* 2009; Olivia *et al.,* 2010; Makunthakumar *et al.,* 2013; Kiran *et al.,* 2015; Nair, 2016). This method has several advantages in the simplicity of technique, fast process, only requires a small amount of DNA samples (0.5-50 ng), and no need initial genome information (Demeke and Adams, 1994; Yu and Pauls, 1994).

The present study aims to analyze the genetic diversity, grouping and genome inference of Pisang Raja local cultivars from several regions of Java Island based on RAPD molecular markers. The results of this study are expected to support the process of characterization and genome identification of Pisang Raja more accurately, which then can be used as information to confirm and evaluate the taxonomy nomenclature of Pisang Raja. It is also expected to be used as basic information for further researches related to conservation, breeding and development of Pisang Raja particularly in Java Island as valuable genetic resources.

Table 1. Samples of banana cultivars used in this study. R1-R13 = in-group, AA3 and BB1 = outgroup.

No.	Local name	Genome*	Sample code	Collection site in Java Island
1	Raja Bali	ABB	R1	Bantul, Central Java
2	Raja Bandung	ABB	R2	Bantul, Central Java
3	Raja Delima	AAA	R3	Malang, East Java
4	Raja Kisto	AAA	R4	Banyuwangi, East Java
5	Raja Gareng	AAB	R5	Temanggung, East Java
6	Raja Jambe	AAA	R6	Malang, East Java
7	Raja Kriyak	AAA	R7	Temanggung, Central Java
8	Raja Madu	AAA	R8	Banyuwangi, East Java
9	Raja Sereh	AAB	R9	Purworejo, Central Java
10	Raja Seribu	AAB	R10	Jakarta
11	Raja Kutuk / klutuk	AAB	R11	Purworejo, Central Java
12	Raja Lini	AAB	R12	Sukoharjo, Central Java
13	Raja Brentel	ABB	R13	Gunung Kidul, Central Java
14	Monyet (*M. acuminata*)	AAw	AA3	Tuban, East Java**
15	Kluthuk wulung (*M. balbisiana*)	BBw	BB1	Banyumas, Central Java

* genome identity based on morphology
** collection of Purwodadi Botanic Garden

MATERIALS AND METHODS

Materials

Thirteen local cultivars of Pisang Raja and two wild bananas (Table 1) were used in this study. It was collected from Banana Germplasm Garden of Yogyakarta, at Special Region of Yogyakarta. Pisang Raja cultivars were originated from several districts of Java Island, consisting of 8 cultivars from Central Java, 4 cultivars from East Java, and 1 cultivar from Jakarta (Table 1). The genomic group of each Pisang Raja cultivar was previously identified morphologically based on minimal descriptor for bananas (Simmonds, 1959), according to information from the curators, and also from some references (Jumari & Pudjoarinto, 2000; Sukartini, 2007; Wahyuningtyas, 2009; Hapsari, 2014; Hapsari *et al.*, 2015b, Nedha *et al.*, 2017). It comprised of AAA, AAB and ABB genomes (Table 1). In addition, wild bananas of *M. acuminata* (AAw) and *M. balbisiana* (BBw) species were also analyzed for comparison as outgroups. Further, the plant material used was young leaves (furled) which dried with *silica gel* prior to analysis, one sample per cultivar.

DNA Isolation

DNA isolation of thirteen samples was carried out using DNA genome purification kit from the Promega Wizard®, following its manufacturer's procedure for plants. Then, the total genome isolated were confirmed both by quantitative and qualitative tests to determine DNA concentration and purity. Quantitative test with determination of absorbance value at length 260 nm and 280 nm was performed using AE-Nano 200 Nucleic Acid Analyze version 2.0. Good DNA purity level indicated by the value of the *Optical Density* (OD) 260/280 nm ratio approximately 1.8-2.0 (Sambrook *et al.,* 1989). Whilst, the qualitative tests were performed using electrophoresis on 1% agarose gel with the addition of 2 µg/ml *Ethidium bromide* (Etbr) for 30 minutes at a voltage of 80 volts, then photographed on GelDOC UV – Transiluminator (BioRAD). The estimated length of total genomic DNA was measured using a 1-Kb DNA *ladder* markers (Thermo Scientific, California, USA).

RAPD - PCR Analysis

DNA amplification was conducted with PCR Thermocycler using 20 selected RAPD primers from Operon Technology Ltd (Table 2). The PCR reaction mixture was performed with a total volume of 10 µl consisting of 1 µl DNA template (5-25 ng/µl), 1 µl primer OPA 1-20 (10 pmol), 3 µl ddH_2O, and 5 µl PCR master mix (Thermo Scientific, California, USA). The PCR cycle consists of DNA pre-denaturation at 94 °C for 4 minutes, then 45 cycles consist of denaturation at 94 °C for 30 seconds, annealing at different temperatures for each primer according to Table 2 for 30 seconds, extension at 72 °C for 30 seconds and followed by post elongation at 72 °C for 5 minutes. PCR products were then visualized in 1.5% agarose gel electrophoresis in TBE buffer 1X, 2 µl EtBr for 50 minutes at 50 volts and documented with UV transillumilator. DNA ladder 100 bp (Thermo Scientific, California, USA) was used to determine the size of DNA amplification fragments.

Table 2. List of RAPD primers used in this study (OPA 1- OPA 20). MT = melting temperature, A = Adenine, T = Thymine, G = Guanine, and C = Cytosine.

Primer code	Primer nucleotide sequence (5'-3')	MT (°C)	Annealing (°C)	GC composition (%)
OPA-01	5' - CAG GCC CTT C – 3'	36.4	41	70
OPA-02	5' - TGC CGA GCT G – 3'	40.7	45	70
OPA-03	5' - AGT CAG CCA C – 3'	34.3	39	60
OPA-04	5' - AAT CGG GCT G – 3'	35.1	40	60
OPA-05	5' - AGG GGT CTT G– 3'	32.6	37	60
OPA-06	5' - GGT CCC TGA C– 3'	35.2	40	60
OPA-07	5' - GAA ACG GGT G – 3'	33.2	38	60
OPA-08	5' - GTG ACG TAG G – 3'	31.1	36	60
OPA-09	5' - GGG TAA CGC C – 3'	37.4	42	70
OPA-10	5' - GTG ATC GCA G – 3'	33.1	38	60
OPA-11	5' - CAA TCG CCG T – 3'	36.7	41	60
OPA-12	5' - TCG GCG ATA G – 3'	34.0	39	60
OPA-13	5' - CAG CAC CCA C – 3'	37.7	42	70
OPA-14	5' - TCT GTG CTG G – 3'	34.3	39	60
OPA-15	5' - TTC CGA ACC C – 3'	34.2	39	60
OPA-16	5' - AGC CAG CGA A – 3'	38.3	43	60
OPA-17	5' - GAC CGC TTG T – 3'	35.7	40	60
OPA-18	5' - AGG TGA CCG T – 3'	36.2	41	60
OPA-19	5' - CAA ACG TCG G – 3'	34.2	39	60
OPA-20	5' - GTT GCG ATC C – 3'	33.5	38	60

Data Analysis

RAPD molecular data were analyzed based on amplified DNA band products that appeared on the gel by scoring prior to analysis to determine polymorphism. RAPD products which reproducible, well resolved and non-ambiguous were scored manually as '1' for presence and '0' for absence of a fragment. The binary data matrix was tabulated for further analysis. Polymorphism analysis and discriminatory power of each primer were evaluated by means of four parameters include polymorphism information content (PIC), effective multiplex ratio (EMR), marker index (MI), and resolution power (RP) (Laurentin and Karlovsky, 2007).

Cluster analysis to determine the grouping pattern and genome inference of Pisang Raja was calculated by Jaccard's coefficient similarity, using PAST application version 3.15. Multivariate ordination analysis of the principal coordinate analysis (PCoA) also conducted by PAST program with menu options: multivariate ordination-principal coordinates analysis, matrix eigenvalues, and eigenvectors (Hammer *et al.*, 2001). Further, the results of genome inference from RAPD analysis were compared to morphological characters and other molecular methods obtained from previous researches and references.

RESULTS AND DISCUSSION

RAPD profiles Pisang Raja

Results of DNA amplification showed that 12 out of 20 RAPD primers had the capability to produced polymorphic bands of Pisang Raja, specifically OPA 1, OPA 2, OPA 3, OPA 4, OPA 5, OPA 8, OPA 11, OPA 16, OPA 17, OPA 18, OPA 19, and OPA 20. However, some samples of Pisang Raja have not produced any bands or absent on certain primers (Figure 1b-d-f). It may due to the absence of homolog primer sequences information in the genome. The number of DNA amplification bands depended on how primer attached to its homolog at DNA template which noted by the presence or absence of an amplification product from a single locus (Tingey et al. 1994). In addition, it may also have caused by technical errors, amplification process and thermal cycle which were less suitable of certain primers for certain sample. Further analysis for those certain primers and samples of Pisang Raja are required to conduct.

Each pattern of DNA band amplification products is an informative character for describing the construction of genetic diversity for genetic relationships among samples. The amplification products showed high polymorphic bands in 13 Pisang Raja cultivars. It comprised 4.83% monomorphic and 95.17% polymorphic bands. Monomorphic bands that emerged showed no variation in all samples, for example at the primers OPA 1 (800 bp), OPA 4 (400 bp) and OPA 11 (1500

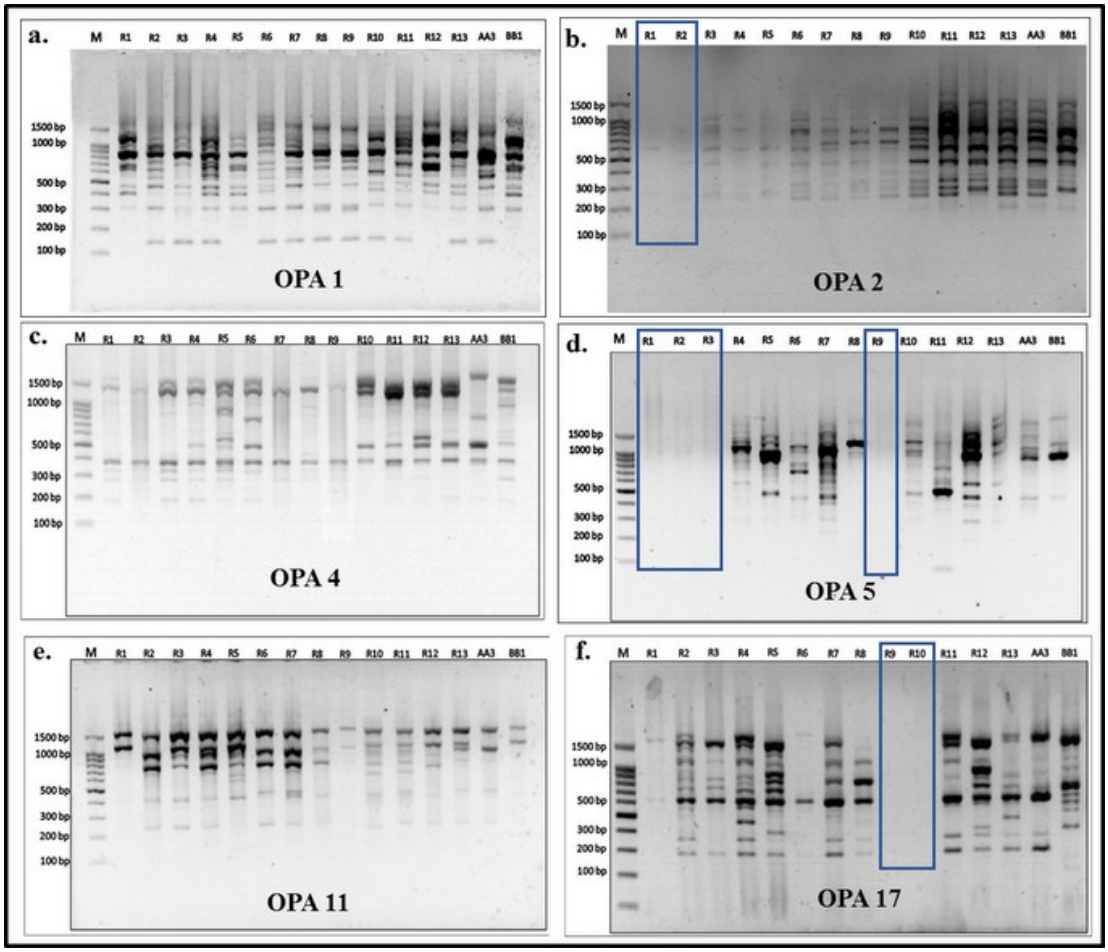

Figure 1. Electrophoregram PCR RAPD which produced DNA polymorphic bands on Pisang Raja. Blue rectangle: showed an absence of DNA band in certain primers and samples.

bp) (Figure 1a-c-e). Those monomorphic bands were presumably considered as part of some genes which encode for the same characters in all Pisang Raja samples.

Further, RAPD primers which produced the highest number of polymorphic bands were OPA 2 (11 bands) and OPA 17 (11 bands), while the lowest polymorphic bands were OPA 1 (8 bands) and OPA 19 (8 bands) (Table 3). RAPD polymorphism is the result of either a nucleotide base change that alters the primer binding site or an insertion or deletion within the amplified region (Williams *et al.* 1990). The differences in polymorphism may be due to the differences in amount of genetic variation that exist among the different accessions (Poerba and Achmad, 2010). Further, the appearance of high polymorphic bands on PCR amplification products indicated that the genetic diversity of the species examined was high (Roldan-Ruiz *et al.*, 2000). Thus, this study provides evidence which indicates that 13 Pisang Raja cultivars examined have high genetic diversity.

Results of polymorphism analysis

The total number of bands (TNB) DNA fragments produced by 12 RAPD primers on 13 Pisang Raja cultivars were 121 bands with sizes ranging from 100 bp to 1500 bp. The highest TNB appeared was produced by OPA 2, OPA 4, and OPA 17; each with 11 bands. Of the 121 total bands produced, 115 of them are considered polymorphic bands. The highest number of polymorphic bands (NPB) produced by OPA 2 and OPA 17 were 11 bands (Table 3). The polymorphic band percentage (PB%) ranged from 80% -100%. Due to their high reproducibility, this result indicated that those 12 primers are suitable to be used as markers for detecting genetic diversity in Pisang Raja. Hence, those 12 RAPD primers (OPA 1, OPA 2, OPA 3, OPA 4, OPA 5, OPA 8, OPA 11, OPA 16, OPA 17, OPA 18, OPA 19, and OPA 20) are proposed as suitable primers for similar research to study the genetic diversity and infer the genome of bananas.

Polymorphism information content (PIC) is information to detect primers which capable of producing polymorphic bands in a population (Roldan-Ruiz *et al.*, 2000). The highest PIC value was indicated by OPA 2 and OPA 17 primers of 0.34, whereas the lowest PIC was indicated by OPA 16 primer of 0.15 (Table 3). PIC maximum value for RAPD markers was 0.5. PIC values were used to

Table 3. Polymorphism analysis results of OPA 1-20 RAPD primers amplification in Pisang. TNB = total number of bands; NPB = number of polymorphic bands; PB = polymorphic band percentage; PIC = polymorphism information content; EMR = effective multiplex ratio; MI = marker index; RP = resolution power.

No	Primer name	TNB	NPB	PB (%)	PIC	EMR	MI	Rp
1.	OPA 1	10	8	80	0.21	80	17.70	15.60
2.	OPA 2	11	11	100	0.34	121	41.02	11.33
3.	OPA 3	10	9	90	0.25	90	22.86	13.07
4.	OPA 4	11	9	82	0.22	99	21.75	9.33
5.	OPA 5	10	10	100	0.27	100	26.54	6.53
6.	OPA 8	9	9	100	0.25	81	20.57	5.07
7.	OPA 11	10	9	90	0.25	90	22.40	10.27
8.	OPA 16	7	7	100	0.15	49	7.40	2.67
9.	OPA 17	11	11	100	0.34	121	40.87	12.80
10.	OPA 18	10	10	100	0.31	100	30.86	8.67
11.	OPA 19	8	8	100	0.20	64	12.52	4.80
12.	OPA 20	9	9	100	0.28	81	22.32	8.00
	Total	121	115	1142	3.19	1155	328.11	117.74
	Mean	10.10	9.58	95.17	0.27	96.25	27.34	9.81

consider which the best primer in RAPD markers and reflects allele diversity and frequency among samples. The higher PIC value means the better of the primer to be used to analyze genetic variation (Roldan-Ruiz *et al*., 2000).

Furthermore, the highest effective multiplex ratio (EMR) value was observed in OPA 2 and OPA 17 (121), and the lowest was observed in OPA 16 (49) (Table 3). EMR analysis was performed to determine the effective ratio of the number of bands produced with the number of polymorphic bands (Roldan-Ruiz *et al*, 2000). While, the OPA 2 primer has the highest marker index (MI) of 41.02, and OPA 16 has the lowest MI value of 7.40. The marker index (MI) value was used to estimate the usefulness of markers in practical terms results in a total band value that appears proportional to the number of polymorphism bands (Varshney *et. al*., 2007). Resolution power (Rp) analysis was used to determine the effectiveness of the primer of produced bands. Each primer has a value of RP which ranged from 2.67-15.60 with an average of 9.81 per primer. The highest RP value was generated by OPA 1 primer with a value of 15.60 while the lowest value was found in OPA 16 primer with a value of 2.67 (Table 3). Based on the overall polymorphism parameters analyzed, the most effective primer in producing polymorphic bands in Pisang Raja was OPA 2 and OPA 17.

Clustering and genome inference of Pisang Raja in Java Island by RAPD marker

Genetic similarity analysis showed that among 13 Pisang Raja cultivars in Java Island has high genetic diversity with similarity coefficient ranged of 0.22-0.61. Low similarity coefficient indicates that the genetic relationship among cultivars observed has high genetic diversity, vice versa. The lowest similarity coefficient was obtained among Pisang Raja Bali (ABB) and Raja Seribu (AAB). They both have different genomes and collected from different provinces *i.e.* Central Java and DKI Jakarta, respectively. Whilst, the highest similarity coefficient was observed between Pisang Raja Kutuk (AAB) and Raja Brentel (AAB), they shared high genetic identity at 0.61 similarities. They both have the same genomes and similar morphological characteristics also collected from the same province, i.e. Central Java.

The conventional classification of banana genotypes into distinct genome combinations by Simmonds and Shepherd (1955) is basically according to their morphological similarity to *M. acuminata* and *M. balbisiana*. The 13 Pisang Raja cultivars examined in this study using RAPD bands profiles resulted a dendogram which may give a picture of genetic relationship and taxonomic position, also genomic group inference of each cultivar which clustered accordingly to their hypothetical genetic homologies. The dendogram was separated into three main clusters at a genetic similarity coefficient of 0.39 (Figure 2). However, the deeper separation of each cluster for genome inference was unclear. It may due to the intensity and number of the amplified DNA bands were less consistent and reproducible on some samples. The first amplification results do not always produce a band with the same intensity at the next amplification. Amplification of each RAPD primer is strongly influenced by the primer attachment site in the DNA template. RAPD primer has amplified whole genomic DNA template, it is possible for primers attachment to be initiated in several places (randomly), but only a few sets can be detected as

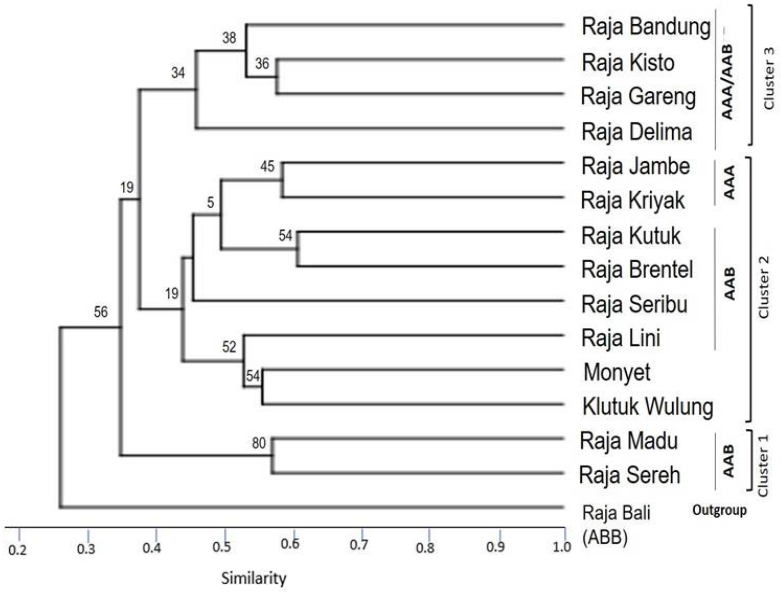

Figure 2. Dendrogram grouping pattern of Pisang Raja and genome inference based on RAPD marker

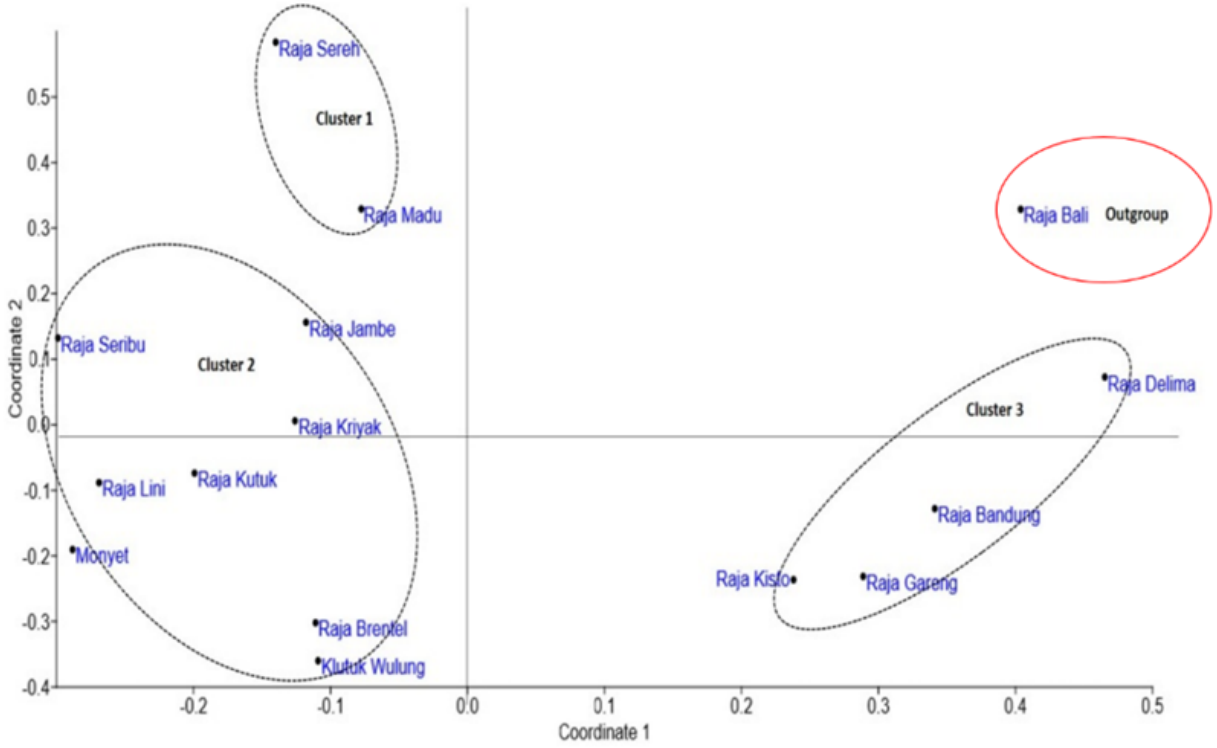

Figure 3. Principal Coordinate (Pco) scatter plot of Pisang Raja based on RAPD marker

after amplification band (Williams *et al*., 1990). As a result, the polymorphic DNA bands produced by each primer differed in the second amplification, both in the size of the number of base pairs and the number of DNA bands, thus affecting the accuracy in grouping banana cultivars with the same genome (Poerba and Ahmad, 2013; Sukartini, 2008).

Cluster 1 consists of Pisang Raja Madu and Raja Sereh which have AAB genomes, with a similarity coefficient of 0.58. Cluster 2 consists of combination both AAA and AAB, with a similarity coefficient of 0.53-0.61; include Pisang Raja Jambe (AAA), Raja Kriyak (AAA), Raja Klutuk (AAB), Raja Brentel (AAB), Raja Seribu (AAB), and Raja Lini (AAB). Likewise, Cluster 3 also consists of combination both AAA and AAB, with a similarity coefficient of 0.43-0.58; include Pisang Raja Kisto (AAA), Raja Delima (AAA), Raja Bandung (AAB) and Raja Gareng (AAB). Surprisingly, Pisang Raja Bali (ABB) was positioned as an out-group with the lowest similarity coefficient of 0.22. It may due to the small amount of polymorphisms obtained from the amplification of several RAPD primers in Pisang Raja Bali sample. Whilst, Pisang Monyet (AAw) and Kluthuk Wulung (BBw), the wild relatives of banana cultivars (previously assumed to be the outgroups)

Table 4. Genome inference comparison of Pisang Raja based on morphology, ITS PCR-RFLP, Microsatellite and RAPD markers.

No.	Pisang Raja	Morphology*	ITS PCR-RFLP **	Microsatellite***	RAPD (this study)
1	Raja Bali	ABB	ABB	ABB	ABB
2	Raja Bandung	ABB	ABB	ABB	AAB
3	Raja Delima	AAA	-	-	AAA
4	Raja Kisto	AAA	AAB	-	AAA
5	Raja Gareng	AAB	-	-	AAB
6	Raja Jambe	AAA	AAA	-	AAA
7	Raja Kriyak	AAA	-	AAA	AAA
8	Raja Madu	AAA	AAA	-	AAB
9	Raja Sereh	AAB	-	AAB	AAB
10	Raja Seribu	AAB	AAB	AAB	AAB
11	Raja Kutuk	AAB	-	-	AAB
12	Raja Lini	AAB	AAB	-	AAB
13	Raja Brentel	ABB	ABB	-	AAB
14	Monyet	AAw	AAw	AAw	AAw
15	Kluthuk Wulung	BBw	BBw	BBw	BBw

* Reference genome based on morphology: Jumari & Pudjoarinto, 2000; Sukartini, 2007; Wahyuningtyas, 2009; Hapsari, 2014; Hapsari *et al*., 2015b, Nedha *et al*., 2017.

** Reference genome based on PCR-RFLP ITS: Ekasari *et al*., 2012; Hapsari *et al*., 2015a; Hapsari *et al*., 2018.

*** Reference genome based on Mikrosatelit: Wahyuningtyas *et al*., 2009; Retnoningsih *et al*., 2010.

were reveal nested in Cluster 2, with a similarity coefficient of 0.56.

PCo analysis was conducted in order to confirm the grouping pattern of Pisang Raja based on clustering analysis. Result of PCo scatters plot diagram was also grouped into 3 main clusters according to its genomes. The diagram was providing a clearer picture ordination of 13 Pisang Raja examined (Figure 3). The grouping patterns based on RAPD markers in this study was presumably caused by genetic differentiation among Pisang Raja cultivars. Some of the factors may cause genetic differentiation, such as geographical isolation and habitat fragmentation (external), as well as internal factors such as mutation, natural selection, genetic drift, and gene flow (Slatkin, 1987).

Genome inference comparison of Pisang Raja based on morphology, ITS PCR-RFLP, microsatellite and RAPD Markers.

Upon this study, RAPD markers are able to reveal the high genetic diversity among Pisang Raja from Java Island as indicated by a high level of polymorphisms. Nonetheless, the grouping pattern for the purpose of genome inference on some bananas was considered moderate in accuration and consistency. This study showed that several Pisang Raja were confirmed to have the same genome identity as the morphological results, such as Pisang Raja Kisto (AAA), Raja Delima (AAA), Raja Jambe (AAA), Raja Kriyak (AAA), Raja Gareng (AAB), Raja Sereh (AAB), Raja Kutuk (AAB), Raja Seribu (AAB), Raja Lini (AAB), and Raja Bali (ABB). However, some cultivars show different results, include Pisang Raja Bandung, Raja Madu, and Raja Brentel (Table 4).

In addition, genome inference from this study compared to other molecular methods such as ITS PCR-RFLP and microsatellite showed same results on some cultivars and also differed on the others (Table 4). For example, Pisang Raja Bali and Raja Madu were confirmed ABB and AAA respectively, according to all molecular methods. Pisang Raja Kriyak was confirmed AAA, both using RAPD and microsatelite. Pisang Raja Lini was confirmed AAA, both using RAPD and ITS PCR-RFLP. Whilst, Pisang Raja Kisto was inferred AAB according to ITS PCR-RFLP but AAA according to RAPD (Table 4).

Further, Pisang Raja Bandung in this study was identified as AAB genome, whereas according to morphological characterization and other molecular methods (ITS PCR-RFLP and microsatellite) was considered as ABB. Likewise, Pisang Raja Madu was considered as AAA by morphology and ITS PCR-RFLP, whereas in this study it was confirmed as AAB. Meanwhile, Pisang Raja Brentel according to morphological characterization by Nedha *et al.* (2017) upon sample from Kediri (East Java) was identified as ABB, whereas in this study upon a sample from Gunung Kidul (Central Java) was inferred as AAB. On the other hand, Hapsari *et al.* (2015b) concluded that Raja Brentel Warangan banana collected from the Yogyakarta region identified as AAB genome; and Raja Prentel (spelling variation) from Pasuruan (East

Java) identified as ABB genome.

The difference results in genome inference based on RAPD marker in this study was suspected because of the band absence on some Pisang Raja cultivars at certain primers (was not successfully amplified), thus the grouping pattern and the genome inference became less precise. This result was supported by the previous study by Sukartini (2008) which shows that some AAB cultivars were not clustered in one group, due to the small amount of polymorphism obtained from the amplification of each RAPD primer. In addition, the locality where was the material collected also presumably as a causing factor. Possibly, the material being studied was actually different. Indeed, the presence of numerous cultivar names and synonyms in different languages and dialects of the region become taxonomic problems. The same cultivars are known by different names in a different region. Occasionally, the same name is applied to distinct cultivars. Phonetic variations associated with tonal languages in Java often result to differences in spelling (Valmayor *et al*., 2000).

Each molecular method in predicting genomes of bananas has advantages and disadvantages, and also showed some inconsistency results. Genome inference using morphological approach has high subjectivity, morphological characters are influenced by environmental factors so that it influences the lack of result validity (Jumari & Pudjoarinto, 2000). The protocol of RAPD method is relatively simple, efficient, and will produce many fragments of DNA so that it can determine the level of polymorphism among organisms with good results. Further, RAPD method uses random primers which amplification processes may be initiated in several places and are dominant so that polymorphic DNA bands results are sometimes inconsistent, differing in band sizes and numbers. In addition, the use of RAPD markers can also provide different results if repeated (Demeke & Adams, 1994; Simpson, 2006; Poerba & Ahmad, 2013). Likewise, the microsatellite method has protocol similar to RAPD but uses specific primers that amplify only at one particular site, and are co-dominant with more informative and accurate results, however the lack of this method is that it requires high costs to design a new primer for specific organism (Wahyuningtyas *et al*., 2009; Retnoningsih *et al*., 2010). Meanwhile, ITS PCR-RFLP method uses even more specific marker, i.e. ITS region which provide more precise results of genome inference, but are technically more difficult because amplification results need to be incubated with specific restriction enzymes of *Rsa*I (Ekasari *et al*., 2012; Hapsari *et al*., 2015a; Hapsari *et al*., 2018).

Inference grouping of Pisang Raja genomes using RAPD markers in this study compared to other methods (morphology, ITS PCR-RFLP, microsatellite) was considered moderately accurate since some cultivars showed same results and also differed on the others. The genomic grouping in this study is the resulted of amplification of all RAPD primers which have not been specifically screened for primers which are suitable for banana cultivars (genome donor A) and (genome donor B). However, RAPD markers have several advantages that are relatively simple, fast, reliable, and quite accurate for checking polymorphisms and genetic variations of bananas to develop further policy on the breeding program and conservation (Pillay *et al*., 2000; Williams *et al*., 1990). The use of RAPD markers has some weakness in grouping the genomes of Pisang Raja (in this study), therefore further research with different techniques of more specifics markers is needed to get more specific and accurate results are necessary. Proposed other methods which more specific but complicated procedures are through ploidy analysis and nuclear DNA content with flow cytometry (Dolezel *et al*., 1997; Asif *et al*., 2001). In addition, there are other more specific molecular methods, i.e. trnL- F, rbcl, rflp, ITS

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CONCLUSIONS

RAPD analysis of 13 Pisang Raja cultivars from Java Island showed high genetic diversity. About 12 out of 20 primers were successfully produced polymorphic bands. Polymorphism analysis showed that the 12 primers (OPA 1, OPA 2, OPA 3, OPA 4, OPA 5, OPA 8, OPA 16, OPA 17, OPA 18, OPA 19, and OPA 20) were suitable and proposed to be used as markers for detecting genetic diversity in Pisang Raja, with OPA 2 and OPA 17 primers being the most effective primers. Pisang Raja cultivars examined were grouped into 3 main clusters following their genomes, namely AAA, AAB, and ABB at genetic similarity coefficient of 0.39. Molecular study of Pisang Raja using RAPD markers was able to describe the genetic diversity among Pisang Raja, however, the grouping pattern for the purpose of genome inference was considered moderate in accuracy and consistency. Therefore, further research using more specific genetic markers are needed to confirm the genome identity of Pisang Raja accurately.

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